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Reversal of Pathology in the Entire Brain of Mucopolysaccharidosis Type VII Mice after Lentivirus-Mediated Gene Transfer

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ABSTRACT

Gene transfer vectors derived from human immunodeficiency virus (HIV-1) efficiently transduce nondividing cells and remain stably integrated in their genome. Long-term expression of reporter genes has been documented after intracerebral injection of these vectors. Using a HIV-based vector, we looked for a reversal of brain damage in the β-glucuronidase-deficient mucopolysaccharidosis type VII mouse, an animal model of human lysosomal storage diseases. The vector suspension was injected stereotactically in the brain of 10-week-old animals, an age at which storage lesions are patent in glia, perivascular cells, and neurons. Either a single intrastriatal injection or multiple injections in both cerebral hemispheres and in the cerebellum were performed. Local tolerance, enzyme delivery, and correction of storage lesions were investigated by comprehensive analysis of serial sections of the entire brain of mice killed 6 or 16 weeks postinjection. Histochemical staining detected enzyme activity in widely distributed areas, the size of which increased with time. Clearance of lysosomal storage extended far beyond enzyme-positive areas. In mice receiving multiple injections of the vector, complete correction or significant reduction of the pathology was observed in every section, suggesting disease regression in the entire brain. These results may have implications for the treatment of neurological symptoms in lysosomal storage diseases.

OVERVIEW SUMMARY

Most lysosomal storage diseases induce severe neurological alterations. Enzyme replacement therapy, which is a promising therapeutic approach for some of these diseases, will not be effective in the brain because of the impermeability of the blood–brain barrier. Methods for direct enzyme delivery throughout the central nervous system are therefore highly desirable. The present study investigates this issue in a mouse model of mucopolysaccharidosis type VII. Secretion of β-glucuronidase, the missing enzyme in this disease, was obtained by stereotactic injection of an HIV-based vector in one or several locations within the brain. Comprehensive analysis of serial sections revealed widespread distribution of the enzyme and complete or partial correction of lysosomal storage lesions. In animals treated with several intracranial injections, improvement was visible in every tissue section, suggesting that reversion of histological lesions can be obtained in the entire brain. These results have potential implications for the treatment of brain damage associated with lysosomal storage diseases.

INTRODUCTION

LYSOSOMAL STORAGE DISEASES form a group of more than 30 metabolic disorders in which the function of one or several lysosomal hydrolases is deficient. The accumulation of intermediate catabolites in lysosomes progressively alters cell function and survival. Although deficiencies affect every tissue, clinical expression varies depending on the missing enzyme. Neurological symptoms are often predominant. Histopathology reveals characteristic vacuolizations in neurons, glia, and perivascular cells. Other frequent symptoms include hepatomegaly, skeletal abnormalities, corneal clouding, and respiratory, cardiac, or renal dysfunctions leading to premature

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death. Because cells can capture extracellular lysosomal enzymes through the mannose 6-phosphate receptor pathway, cross-correction of the deficiency can be achieved by the infusion of purified enzyme, as illustrated by the regression of hepatomegaly in Gaucher disease patients receiving glucocerebrosidase (Grabowski *et al.*, 1998). Enzyme replacement therapy will likely by proposed for the treatment of other lysosomal storage diseases in the near future. However, clinical efficacy will require a sustained and widespread enzyme delivery to the entire brain. The impermeability of the blood–brain barrier to lysosomal enzymes is a critical issue with that respect. Since disruption of the blood–brain barrier cannot be performed at each enzyme infusion, the success of these treatments will rely on direct enzyme supply to the CNS. A possible approach with that goal in mind is the genetic engineering of brain cells in order to induce local enzyme secretion.

Gene transfer with integrative vectors offers the possibility of permanent expression in target cells. Unlike retroviral vectors derived from murine leukemia viruses, lentiviral vectors integrate into the genome of nondividing cells (Naldini *et al.*, 1996a,b; Reiser *et al.*, 1996; Blömer *et al.*, 1997; Miyoshi *et al.*, 1997). Expression of reporter genes introduced via lentiviral vectors was documented in rat neurons, photoreceptor cells, and retinal pigment epithelium (Naldini *et al.*, 1996b; Miyoshi *et al.*, 1997). Vector injections seemed well tolerated. The absence of inflammatory reaction at the injection site and the tolerance of transduced cells by the immune system ensure sustained expression over several months and make these vectors more suitable than adenovirus vectors for future clinical application in the CNS. Pseudotyping of lentiviral vectors with the vesicular stomatitis virus envelope G protein (VSV-G) increases resistance of the particles and favors neuron infection over other cell types (Blömer *et al.*, 1997). Constant improvements of vector design have decreased safety concerns regarding the use of this material for human gene therapy (Zufferey *et al.*, 1997, 1998). Optimization of vector production and stereotactic delivery methods allowed efficient gene transfer in the brain of nonhuman primates (Kordower *et al.*, 1999) and expression of therapeutic genes in neurons (Déglon *et al.*, 1998; Blömer *et al.*, 1999), thus demonstrating the potential effectiveness of lentiviral vectors for treating neurodegenerative diseases and metabolic disorders affecting the CNS.

Mucopolysaccharidoses (MPSs) are a subgroup of lysosomal storage diseases in which skeletal development is affected and severe mental retardation is highly prevalent. We investigated the potential of lentivirus vectors for treating brain damage in a mouse model of MPS VII (Birkenmeier *et al.*, 1989), a disorder due to a genetic alteration of the β-glucuronidase gene. Affected mice were treated as adult, an age at which pathology is already severe in the brain. A human immunodeficiency virus (HIV)-based vector encoding human β-glucuronidase was injected stereotactically in the brain. Either a single injection in the striatum, or three injections in each hemisphere plus one in the cerebellum, were performed. We carefully monitored gene transfer, enzyme diffusion, and histological correction in serial sections of the whole brain of animals killed 6 or 16 weeks after injections. Data indicate that lentiviral vectors can be used to improve lysosomal storage lesions in the entire brain of MPS VII mice.

MATERIALS AND METHODS

Mice

Heterozygous ($Gus^{mps/+}$) mice (Birkenmeier *et al.*, 1989) obtained from E. Birkenmeier at The Jackson Laboratory (Bar Harbor, ME) were bred and mutants ($Gus^{mps/mps}$) were identified at weaning age by the absence of β-glucuronidase activity in trail clip homogenates.

Construction and production of HIVβgluc

The gene transfer plasmid pHR'hβgluc, flanked by HIV long terminal repeats (LTRs), contains the human β-glucuronidase cDNA inserted downstream of the cytomegalovirus (CMV) enhancer and promoter elements. pHR'hβgluc was constructed by digesting pHR'CMVLacZ (Naldini *et al.*, 1996a) with *Bam*HI and *Xho*I and inserting the human β-glucuronidase cDNA, flanked by *Bcl*I/*Xho*I restriction sites, in the place of the *lacZ* gene. HIV vectors were prepared as previously described (Naldini *et al.*, 1996a) by cotransfecting 293T cells with a three-system plasmid including (1) pHR'hβgluc, (2) a second-generation packaging plasmid (p8.91) (Zufferey *et al.*, 1997), and (3) the VSV-G envelope protein expression plasmid (pMDG). In control experiments, pHR'CMVLacZ (Naldini *et al.*, 1996a), which encodes *Escherichia coli* β-galactosidase, replaced pHR'hβgluc. Supernatant was collected every 12 hr for 2 days. HIV Gag protein p24 measured in vector preparations by enzyme-linked immunosorbent assay (ELISA) (Du Pont, Wil-mington, DE) was routinely in the range of 200–400 ng/ml. Preparations were concentrated 100-fold by pelleting at 50,000 $\times$ *g* and resuspending in serum-free culture medium. Vectors were titered on D17 dog fibroblasts by serial dilutions of the concentrated preparation. Colony-forming units (CFU) were scored at 48 hr by histochemical staining of human β-glucuronidase activity after heat inactivation of canine endogenous enzyme. The titer of the concentrated HIVβgluc vector preparation was 4×10^7 CFU/ml with a ratio of 2500 CFU/ng of p24. Searches for replication-competent particles by cocultivation of MT4 lymphocytes exposed to the vector with HeLa P4 cells, (Dragic *et al.*, 1992) were negative.

Intraparenchymal injections

Mice were anesthetized with ketamine/xylazine (0.1/0.01 mg/g of body weight) and mounted onto a stereotactic frame (David Kopf Instruments, Tujunga, CA), and the skull was exposed by a small incision. Single injections in the striatum were performed at coordinates +0.2 mm anterior to bregma, 2.0 mm lateral to the midline, and 4.5 mm depth. Multiple injections were performed at the following coordinates: in hemispheres, +2.0 mm anterior to bregma, 1.0 mm lateral to the midline, 3.0 mm depth; +0.2 mm anterior to bregma, 2.0 mm lateral to the midline, 4.0 mm depth; −2.0 mm posterior to bregma, 1.5 mm lateral to the midline, 3.5 mm depth; in the cerebellum, −6.0 mm posterior to bregma, 0.0 mm lateral to the midline, 3.0 mm depth. Five microliters of virus were loaded into a Hamilton syringe mounted to the stereotactic frame and delivered with an ultramicropump (World Precision Instruments,

Hertfordshire, UK) at a rate of 0.5 μl/min through a 30-gauge needle at each injection site. The needle was brought up 0.5 mm after injection of 2.5 μl and the rest of the volume was injected. The needle was slowly withdrawn after an additional 5 min, the scalp was closed, and the animals were returned to recovery cages.

Quantification of lysosomal enzyme and β-galactosidase activities in brain extracts

Deeply anesthetized animals were killed and perfused with phosphate-buffered saline (PBS). The skull and cerebellum were removed and each cerebral hemisphere was stored separately at −80°C. Tissues were homogenized in lysis buffer (0.02 *M* Tris, 0.075 *M* NaCl, pH 7.5) and centrifuged at 12,000 rpm for 10 min, at 4°C. Four milligrams of tissue was assayed in a fluorimeter (TKO100; Hoefer, San Francisco, CA) for β glucuronidase or β-hexosaminidase activity, using 10 m*M* 4-methylumbelliferyl-β-D-glucuronide (Fluka, Buchs, Switzerland) or 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (Sigma, St. Louis, MO) as substrate, respectively (Glaser and Sly, 1973). β-Galactosidase activity in extracts was assayed with a Galacto-Light Plus kit (Tropix, Bedford, MA).

Detection of β-glucuronidase histochemistry in tissue sections

Animals were anesthetized and perfused with 4% paraformaldehyde–1% glutaraldehyde. Brains were removed and postfixed in the same fixative for 5 hr. Serial 100-μm coronal sections were prepared with a Vibratome (Leica [Bensheim, Germany]; total, 60–80 sections per brain). One section of every five ($n = 12$–17; i.e., 500 μm apart) was stained for β-glucuronidase activity by overnight incubation at 37°C with 0.004% hexazotized pararosaniline in 0.25 m*M* naphthol-AS-BI-β-D-glucuronide (Fluka), as described (Hayashi *et al.*, 1963).

Assessment of lysosomal storage correction

Sections located at a maximal distance of 200 μm from those examined by β-glucuronidase activity (the adjacent section or the next one) were postfixed with 1% paraformaldehyde–2% glutaraldehyde followed by 1% osmium tetroxide and used to prepare semithin sections. Fragments located in the striatum or in various locations in the cortex were embedded in Epon/Araldite, and 1-μm-thick sections were cut with an ultramicrotome and stained with toluidine blue for 30 sec in sodium carbonate, pH 7.2.

In situ *hybridization*

Animals were anesthetized and perfused with 4% paraformaldehyde, after which brains were removed and postfixed for 16 hr in the same fixative. After cryoprotection with 30% sucrose, tissues were embedded in O.C.T. medium (Miles, Elkhart, IN). Ten micromoles coronal cryosections were prepared and postfixed with 4% paraformaldehyde. A 370-bp probe containing the 3′ end of human β-glucuronidase cDNA was obtained as published elsewhere (Ghodsi *et al.*, 1998). Antisense cRNA was constructed as described (Simmons *et al.*, 1989). Briefly, *in vitro* transcription was carried out for 1 hr at 37°C using 1 μg of linearized cDNA, 1× transcription buffer (40 m*M* Tris [pH 7.9], 6 m*M* $MgCl_2$, 2 m*M* spermidine, and 10 m*M* NaCl), ATP, CTP, and GTP (200 μ*M* each), 37.7 U of RNAguard (Amersham, Arlington Heights, IL), 100 μCi of [α-^{33}P] UTP (Amersham), and 20 U of SP6 RNA polymerase (Promega, Madison, WI). The reaction was stopped with 10 U of DNase I (Promega) for 15 min at 37°C. The probe was then hydrolyzed for 20 min at 60°C and purified through a chromatography column (Clontech, Palo Alto, CA). Hybridization was performed overnight at 50°C using 50,000 cpm per slide. After stringent washing, slides were exposed to Kodak (Rochester, NY) NTB2 emulsion for 10 to 15 days. Once developed, slides were counterstained with hematoxylin–eosin. Sense cRNA hybridization was performed under identical conditions with T7 RNA polymerase (Promega) instead of SP6 (not shown). Adjacent slides were stained for β-glucuronidase activity as described above.

PCR

Genomic DNA was extracted from tissues by standard procedures. HIVβgluc vector genomes were detected by nested polymerase chain reaction (PCR) amplification of a 466-bp fragment of the human β-glucuronidase cDNA (positions 917 to 1383), using the following primer couples: first amplification, 5′-CAGCTGACTGCACAGACGT-3′/5′-GTAGCCAGCAGATTCTAGGTG-3′; second amplification, 5′-CTGTGTCTGACTTCTACACA-3′/5′-ATGCAGAGAAACGTTGTTGAAG-3′. Amplification was performed with *Taq* polymerase (Perkin-Elmer, Norwalk, CT); DNA, 500 ng; primers, 25 μ*M*; $MgCl_2$, 25 m*M*. Amplification products were revealed by Southern blotting, using a ^{32}P-labeled probe of 679 bp obtained by *Bam*HI digestion of the human β-glucuronidase cDNA (positions 974 to 1653).

RESULTS

Single vector injection in the striatum

In a preliminary experiment, we measured enzyme activity in brain extracts of MPS VII mice after a single stereotactic injection of HIVβgluc. Ten 10-week-old MPS VII mice received 2×10^5 infectious particles (80–100 ng of p24) into the right striatum. Animals were killed 1 or 6 weeks later and each injected hemisphere was homogenized separately and assayed for β-glucuronidase activity. Values were compared with those of heterozygous mice, which have a normal phenotype (Fig. 1). Mean β-glucuronidase activity in the injected hemisphere corresponded to 30 and 100% of that of heterozygous mice at 1 and 6 weeks, respectively. Activity measured at 6 weeks in contralateral hemispheres was equivalent to 25% of heterozygous mouse levels. β-glucuronidase deficiency is associated with secondary elevations of several lysosomal enzymes, including β-hexosaminidase. Analysis of β-hexosaminidase in brain extracts of treated mice showed that secondary elevation of this enzyme was partially reverted (Fig. 1). These results demonstrated a reduction in the disequilibrium between lysosomal enzymes and suggested a therapeutic effect in treated MPS VII mouse brains.

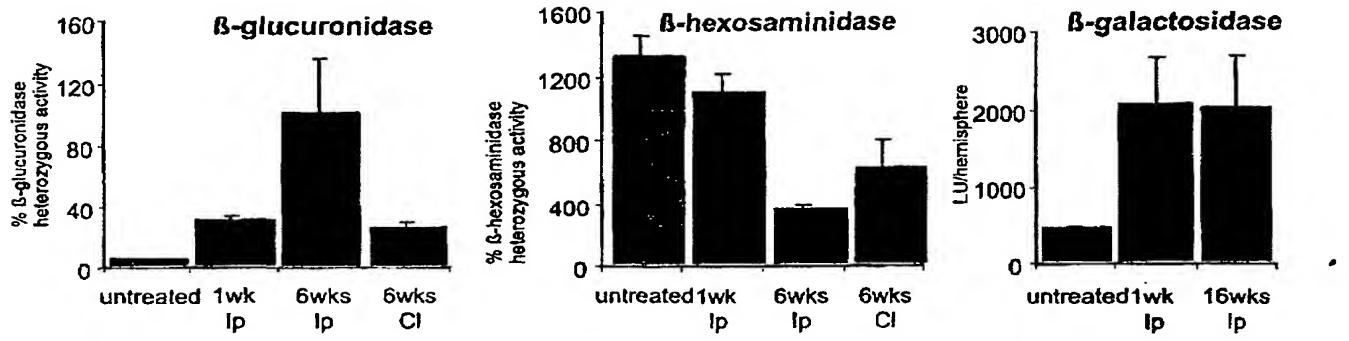

FIG. 1. Enzyme activities in brain extracts. β-glucuronidase, β-hexosaminidase, and β-galactosidase activities were measured in brain homogenates. Extracts were prepared from MPS VII (β-glucuronidase and hexosaminidase) or normal (β-galactosidase) mice, either untreated ($n = 3$) or injected with the HIVβgluc (β-glucuronidase and hexosaminidase) or the HIVβ-gal (β-galactosidase) vector. Homogenates were prepared from the entire injected (Ip) or the contralateral (Cl) hemispheres. Animals were killed 1 week ($n = 6$, 1wk), 6 weeks ($n = 4$, 6wks), or 16 weeks ($n = 4$, 16wks) after a single intrastriatal injection. β-glucuronidase and hexosaminidase data are the mean percentages ± SEM of activity measured in heterozygous mice (β-glucuronidase, 100% = 3.1 ± 0.4 units/mg of protein; β-hexosaminidase, 100% = 106 ± 0.1 units/mg of protein; $n = 2$). β-galactosidase activity is expressed as light units per hemisphere.

We wondered whether the increase in β-glucuronidase activity between weeks 1 and 6 resulted from the uptake and accumulation of the secreted enzyme by neighboring cells, a phenomenon specific for lysosomal enzymes. An HIV-derived vector encoding *E. coli* β-galactosidase, an enzyme for which secretion and cellular uptake has not been documented, was used for that purpose. Normal mice were stereotactically injected in the striatum with 2×10^5 PFU of an HIV-1 vector containing the *E. coli lacZ* reporter gene and killed 1 or 16 weeks later. Whole-brain extracts were prepared and β-galactosidase activity was measured by chemiluminescence (Fig. 1). Data indicated a plateau activity from 1 to 16 weeks after vector injection. These data support the assumption that increasing β-glucuronidase activity between weeks 1 and 6 in animals treated with HIVβgluc resulted from secretion of the enzyme by transduced cells and recapture by neighboring tissues.

To delineate the area of diffusion of human β-glucuronidase and subsequently to investigate the correction of lysosomal storage lesions, adult MPS VII mice (8 to 10 weeks old) were injected as described above and serial 100-μm coronal sections of both hemispheres ($n = 60$) were prepared 6 or 16 weeks later for histochemistry and histology analysis. Extension of β-glucuronidase-positive areas was assessed in each mouse ($n = 3$ for each group). Histochemical staining (Fig. 2) showed that areas positive for β-glucuronidase activity expanded with time, confirming that the enzyme progressively accumulated in brain tissues. Areas positive for β-glucuronidase activity reached a diameter of up to 5.5 mm around the needle track at 16 weeks. Stained surfaces at the injection site were 15.9 and 23.7 mm^2 at 6 and 16 weeks, respectively. The ratio of the brain volume that was stained was estimated as the ratio of the sum of stained surfaces to the sum of total section surfaces. Data indicated staining in 5 and 10% of the total injected hemisphere at 6 and 16 weeks, respectively. Moreover, a few β-glucuronidase-positive cells were detected in the contralateral, noninjected hemisphere (data not shown).

In situ hybridization was performed for the detection of transduced cells expressing human β-glucuronidase mRNAs. Ten-micrometer coronal brain sections of the injected area of the striatum were prepared 6 and 16 weeks ($n = 2$ for each group) after administration of HIVβgluc. Thirty to 50 positive cells were revealed on positive sections (Fig. 3). They were mostly located along the needle track, indicating limited diffusion of the vector. Areas positive for β-glucuronidase activity extended much further away (Fig. 3), in agreement with enzyme secretion in the extracellular environment. The number of β-glucuronidase mRNA-expressing cells was not significantly different at 6 and 16 weeks. Cell transduction and β-glucuronidase cDNA expression levels were therefore stable, at least over 16 weeks.

Whole-brain enzyme delivery after multiple HIVβgluc vector injections

Considering that a single intrastriatal injection of 2×10^5 PFU of HIVβgluc was sufficient to allow enzyme delivery to a significant volume of brain tissue, we examined enzyme delivery after multiple injections. Five adult MPS VII mice received seven stereotactic injections: three in each hemisphere and one in the cerebellum. Injection sites are indicated in a schematized sagittal section of the brain in Fig. 4 (see also Materials and Methods). Each inoculum contained 2×10^5 PFU of HIVβgluc. Animals recovered rapidly and behaved like untreated littermates until killed at 6 weeks ($n = 3$) or 16 weeks ($n = 2$). Serial 100-μm coronal sections ($n = 80$) of the entire brain were made. At least one of every five sections was stained for β-glucuronidase activity ($n = 17$). These sections are indicated in Fig. 4. Activity could be revealed in every analyzed section and in both hemispheres, with the exception of section M (posterior region of the putamen, piriform cortex, anterior lobules of the cerebellum). Examples of positive sections from the brain of a mouse killed 16 weeks after multiple injections

are shown in Fig. 4, as well as the estimated maximal extension of stained areas in this animal. Staining intensity was maximal in the striatum (Fig. 4, sections C to I), but extended to the corpus callosum (Fig. 4, sections C to G), the hippocampus (Fig. 4, section I), the substantia nigra (Fig. 4, section L), and the cortex (Fig. 4, sections, A, C, D, and G). Enzyme activity detected on coronal sections of the cerebellum was centered around posterior cerebellar nodules (Fig. 4, section P). We estimated that the volume of brain tissue staining for β-glucuronidase activity represented 10.4 ± 1.4 and 19.8 ± 3.5% of the whole brain at 6 and 16 weeks, respectively. These data confirmed that enzyme-positive areas progressively expanded with time.

A search for vector genomes was performed by PCR on a group of animals that received multiple vector injections. Three injections were performed in one hemisphere and one in the cerebellum. Animals were killed 16 weeks after injection. DNA was extracted from the whole injected hemisphere, the whole noninjected contralateral hemisphere, the cerebellum, and from various extracranial tissues. Signal specific for vector genome was detected in the injected hemisphere and the cerebellum (Fig. 5). A faint signal was also detected in the contralateral hemisphere in one mouse. No signal was detected in liver, lung, heart, and skeletal muscles.

Correction of lysosomal storage lesions

Sections located between those that had been stained for enzyme activity were used for histopathology studies. For that purpose, sections were postfixed and 1-mm-cubic fragments were randomly picked from the striatum and from one or several locations in the cortex. Semithin sections were prepared from these fragments and stained with toluidine blue.

In untreated MPS VII littermates, extensive lysosomal storage lesions were present in glia, perivascular cells, and neurons at the time of vector injection. On semithin sections stained with toluidine blue, lesions appeared as white inclusions in the cytoplasm, which correspond to distended lysosomes (Fig. 6, cortex–/–, meninges –/–, cerebellum –/–; lesions are indicated with arrowheads). Storage was significantly aggravated 16 weeks later (not shown). Analysis of the brain of treated mice showed a dramatic improvement in lysosomal storage lesions, both in animals killed 6 weeks (not shown) or 16 weeks (Fig. 6) after vector injection. Disappearance of histological abnormalities was observed both in the striatum (not shown) and in the cortex (Fig. 6).

Depending on the area, vacuolization either had completely disappeared or persisted in a fraction of the cells but was significantly reduced in comparison with untreated brain. The average percentage of correction was estimated in every section. One hundred percent means that vacuolization was not observed in any cell in the section. Lower percentages refer to the fraction of cells in which storage lesions were not seen. In areas where β-glucuronidase activity was detected, correction was always complete. In other areas, correction was either complete or partial. Figure 6 shows examples of complete correction in the ipsilateral parietal cortex after a single injection (ipsilateral) and in the rostral cortex after multiple injections (section A), both areas in which enzyme activity was not detected. In areas where correction was partial, the disappearance of vacuolization was more frequently observed in neurons than in glia and perivascular cells. Examples of areas in which correction was estimated at 50% are shown in Fig. 6 (single injection, contralateral; multiple injections, section L).

In animals treated with a single intrastriatal injection of HIVβgluc, complete or partial correction was observed as far as 500 μm away from areas where lysosomal enzyme activity was detected (Fig. 6, distal). Correction levels decreased as the distance from areas where enzyme activity could be revealed increased. A disappearance of vacuolizations was also observed in leptomeninges (Fig. 6, meninges). As indicated above, a 50% correction was seen in the contralateral cortex (Fig. 6, contralateral).

In animals treated with multiple injections of HIVβgluc, improvement of lysosomal storage lesions was observed in sections adjacent to each of the 17 coronal sections of the brain indicated in the sagittal scheme of Fig. 4, including in the cerebellum. Correction was complete in the striatum as well as in other areas where β-glucuronidase activity was detected (not shown). Pictures of almost complete correction (>80%) in the rostral cortex and of partial correction (50%) in the occipital cortex are shown in Fig. 6, sections A and P, respectively. Lesions in the cerebellum of untreated mice affected glial cells preferentially, whereas Purkinje cells remain intact (Fig. 6, cerebellum –/–). A complete (Fig. 6, section P) or almost complete correction of these lesions was seen in treated animals. As an example, Fig. 7 shows the estimated levels of correction at various locations in sections A, F, L, and P of a mouse killed 16 weeks after multiple injection. Correction was not investigated in the brainstem.

DISCUSSION

The β-glucuronidase-deficient mouse has been extensively used as a study model for CNS pathology caused by metabolic disorders. The possibility of a cross-correction of the gene defect by enzyme capture suggests that β-glucuronidase delivery in the brain could restore a normal glycosaminoglycan degradation pathway, thus preventing or even reversing lysosomal storage lesions, which occur early in life. The feasibility of preventing the development of storage lesions in the brain has been confirmed in newborn MPS VII mice engrafted *in situ* with immortalized enzyme-secreting cells (Snyder *et al.*, 1995), or injected intravenously at birth with purified enzyme (Vogler *et al.*, 1993, 1996; Sands *et al.*, 1994, 1997a) or with a recombinant adeno-associated vector encoding β-glucuronidase (Daly *et al.*, 1999a,b). Reversion of preexisting brain lesions in adult animals has also been documented. Transient correction was reported after engraftment of enzyme-secreting primary cells (Taylor and Wolfe, 1997) or *in situ* injection of an adenovirus vector (Ghodsi *et al.*, 1998; Stein *et al.*, 1999). Sustained correction was observed after *in situ* injection of an adeno-associated virus (AAV) vector (Skorupa *et al.*, 1999; Bosch *et al.*, 2000).

The originality of the present study consists in the use of a lentivirus-based gene transfer vector for expressing the human β-glucuronidase cDNA in the brain tissues of MPS VII mice. Stable gene transfer and expression allowed sustained enzyme secretion and delivery to a significant volume of the brain,

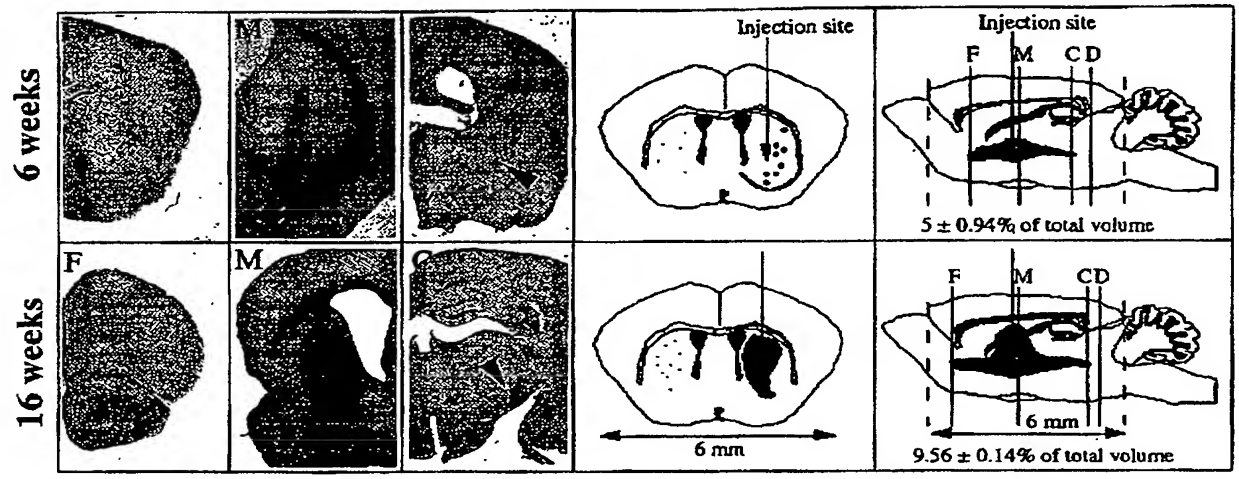

FIG. 2. Distribution of β-glucuronidase activity in MPS VII mouse brains after a single intrastriatal injection of HIVβgluc. Animals were killed 6 (upper row) or 16 (bottom row) weeks after intrastriatal injection of HIVβgluc. Serial 100-μm-thick coronal sections of the brain hemispheres were prepared ($n = 60$). One of every five sections ($n = 12$) was stained for β-glucuronidase activity. Pictures correspond to the most frontal positive section (F), the injection site (M), and the most caudal positive section (C). Arrowheads indicate weakly positive areas. Extension of enzyme-positive areas is represented in schemes of coronal and sagittal brain sections at the injection site. D lines correspond to sections where enzyme activity was not detected, while lysosomal storage lesions were corrected (see Fig. 6). Numbers in red indicate the estimated percentage of the hemisphere volume where β-glucuronidase activity was detected (contralateral hemispheres were not included).

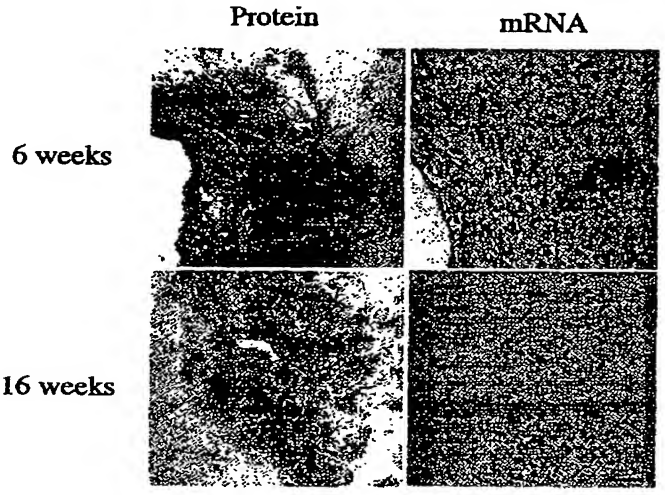

FIG. 3. Detection of human β-glucuronidase mRNAs in brain sections by *in situ* hybridization. Adjacent coronal 10-μm sections of brain hemispheres injected with HIVβgluc, 6 weeks (upper row) or 16 weeks (bottom row) before sacrifice, were treated for the detection of human β-glucuronidase mRNA-expressing cells by *in situ* hybridization (right column) or for the detection of β-glucuronidase activity by histochemical staining (left column). Bar corresponds to 50 μm. Sections stained for mRNA expression and those stained for enzyme activity were located 200 and 30 μm apart at 6 and 16 weeks postinjection, respectively.

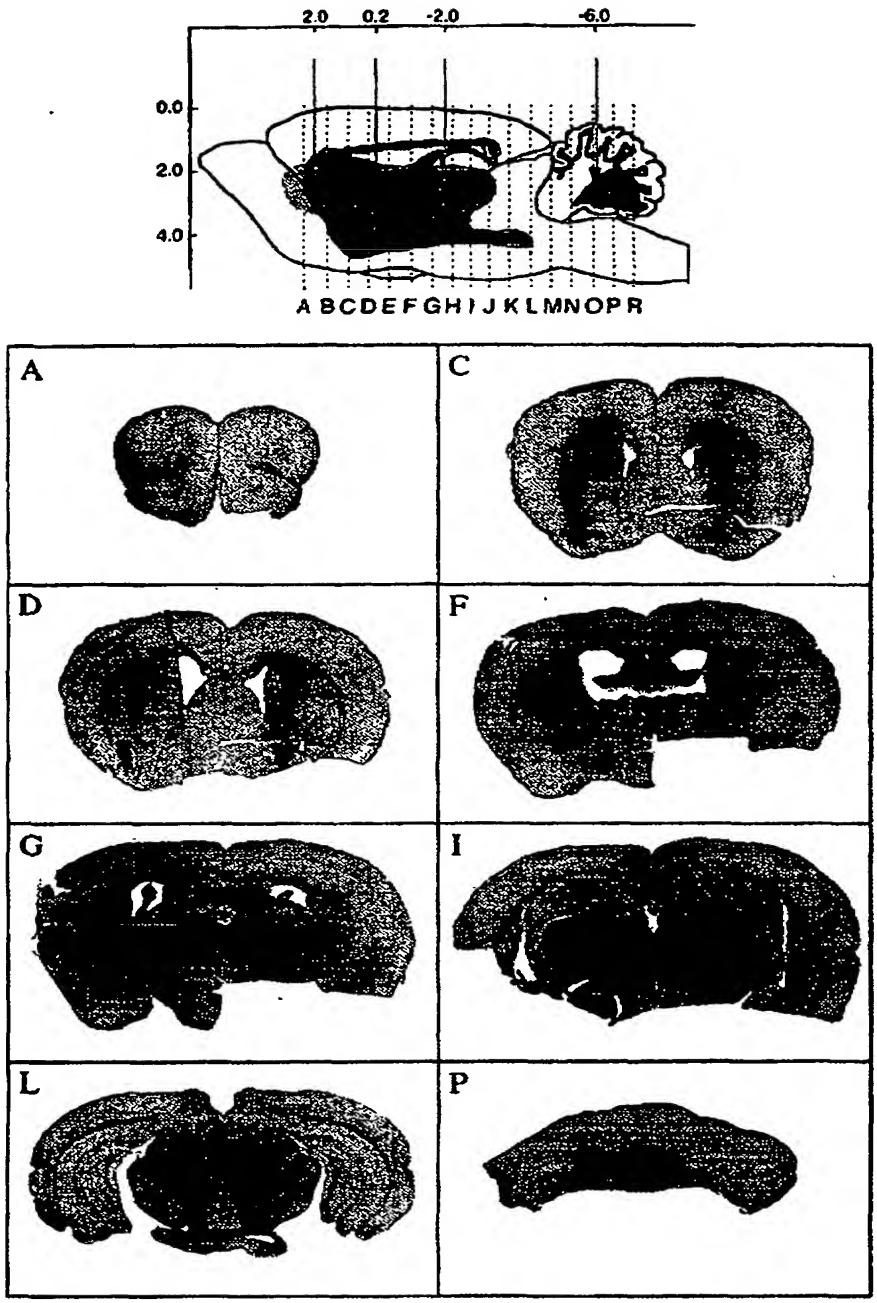

FIG. 4. Distribution of β-glucuronidase activity in MPS VII mouse brain after multiple injections of HIVβgluc. A 10-week-old MPS VII mouse received three injections of HIVβgluc in each hemisphere and one injection in the cerebellum, as indicated in Materials and Methods and in the schematic drawing of a sagittal section of the brain (gray arrows), on which coordinates of the stereotactic injections are shown. The mouse was killed 16 weeks later and serial 100-μm-thick coronal sections of the brain were prepared ($n = 80$). One of every five sections was stained for β-glucuronidase activity ($n = 7$, A to R as indicated as dashed lines on the scheme). Pictures of sections A, C, D, F, G, I, L, and P are shown. The approximate extent of areas stained for β-glucuronidase activity is indicated in red on the scheme. We assume that regions more rostral than section A were likely positive, as indicated by the pink area.

which increased with time. Extended delivery areas after multiple stereotactic injections improved lysosomal storage lesions in every examined location within the entire mouse brain, an efficacy not previously achieved, which is relevant to medical application.

HIV-mediated gene delivery

HIV-mediated gene delivery in the brain has been documented mostly in rats, using reporter genes (Naldini *et al.*, 1996a,b; Blömer *et al.*, 1997; Miyoshi *et al.*, 1997). Studies document gene expression in various brain cell types, pointing out predominant expression in neurons. It has been suggested that the neurotropism of the VSV-G envelope used for pseudotyping vector particles favors neuron transduction. Studies also document sustained expression over long periods of time, as well as the absence of noticeable inflammatory local reaction and immune response against transduced cells. Improvements in vector design, preparation methods, and injection procedures have increased gene transfer efficiency and expression levels significantly (Zufferey *et al.*, 1997, 1998, 1999; Miyoshi *et al.*, 1998). Impressive results obtained in monkeys after several stereotactic injections of an HIV-based vector encoding β-galactosidase (Kordower *et al.*, 1999) and delivery of potentially therapeutic genes in the CNS (Déglon *et al.*, 1998; Blömer *et al.*, 1999) suggest that lentivirus vectors could be effective for the treatment of human neurological disorders.

In situ hybridization studies performed in our MPS VII mice treated with a single injection of HIVβgluc showed human β-glucuronidase mRNA expression in a relatively low number of cells, which were mostly located along the needle track. Although *in situ* hybridization, which is not a highly sensitive detection method, may have failed to reveal expression in a fraction of transduced cells, it is likely that expression levels were suboptimal in our study. A possible explanation would be that HIV-based vectors diffuse or infect cells inefficiently in MPS VII mouse brain because of pathological abnormalities. However, we assume that significantly more efficient gene transfer and higher expression levels can probably be achieved in a similar experimental setting by optimizing vector design, for example, by inserting posttranscriptional regulatory elements from the woodchuck hepatitis virus (WPRE; Zufferey *et al.*, 1999) or a promoter more active in brain cells.

Vector genomes were detected by PCR in the injected cerebral and cerebellar tissues and, at a low level in one mouse, in the noninjected contralateral hemisphere. Although this result suggests that retrograde transport of HIV vector particles may have occurred, we consider that further studies are needed to document this issue more carefully. Interestingly, with respect to safety considerations, the absence of detectable vector genome in extracranial tissues showed that HIV vector particles did not disseminate significantly out of the brain, even after multiple intracranial stereotactic injections.

Lysosomal enzyme delivery in mouse brain

Despite limited gene transfer, we observed a widespread distribution of human β-glucuronidase in the brain of treated MPS VII mice. Enzyme activity could be revealed several millimeters away from the location of transduced cells that express β-glucuronidase mRNAs, and in the contraleral hemisphere. Although numbers of expressing cells remained constant over time, areas in which enzyme activity could be detected increased in size, suggesting progressive accumulation of the protein in tissues. Mechanisms presumably accounting for expansion include secretion of the protein by transduced cells, diffusion in the extracellular matrix, capture by surrounding cells through the mannose 6-phosphate receptor pathway, and retrograde transport of the protein toward lysosomal compartments located in remote neuronal bodies. This secretion–recapture pathway is a property shared by the various lysosomal enzymes. It is therefore expected that other lysosomal enzymes would similarly spread throughout brain tissues. In contrast, delivery of *E. coli* β-galactosidase, which is not secreted and consequently not recaptured, did not increase with time and was presumably restricted to transduced cells. Expansion of β-glucuronidase-positive areas in the brain of MPS VII mice has been reported previously after adenovirus (Ghodsi *et al.*, 1998; Stein *et al.*, 1999) or adeno-associated virus (Skorupa *et al.*, 1999; Bosch *et al.*, 2000)-mediated gene transfer. This phenomenon suggests that delivery of the enzyme to the entire brain could be feasible. We assessed this possibility by injecting HIVβgluc into multiple sites in the brain. Human β-glucuronidase could be detected by histochemical staining in almost every section after three injections in each hemisphere. Injection in the cerebellum also resulted in detectable enzyme activity in tissue sec-

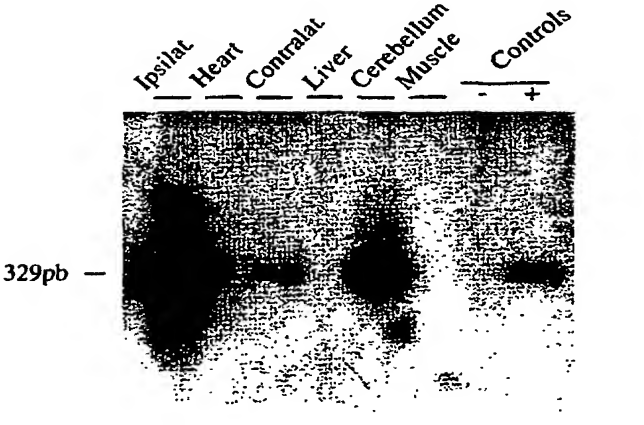

FIG. 5. Detection of vector genome by PCR in brain and peripheral tissues. Shown are data from one MPSVII mouse killed 16 weeks after three injections of HIVβ-gluc in the right hemisphere and one injection in the cerebellum. DNA was extracted from the injected hemisphere (Ipsilat.), the contralateral hemisphere (Contralat.), the cerebellum, and various indicated extracranial tissues. A fragment of the human β-glucuronidase cDNA was amplified. Controls are amplification reactions performed in the absence of DNA (–) and in the presence of 0.1 pg of plasmid DNA encoding the HIVβgluc vector (+; signal generated by 1 vector genome among 400 cells). Amplification products were revealed by Southern blotting, using a human β-glucuronidase-specific ^{32}P-labeled probe. The film was exposed for 4 hr.

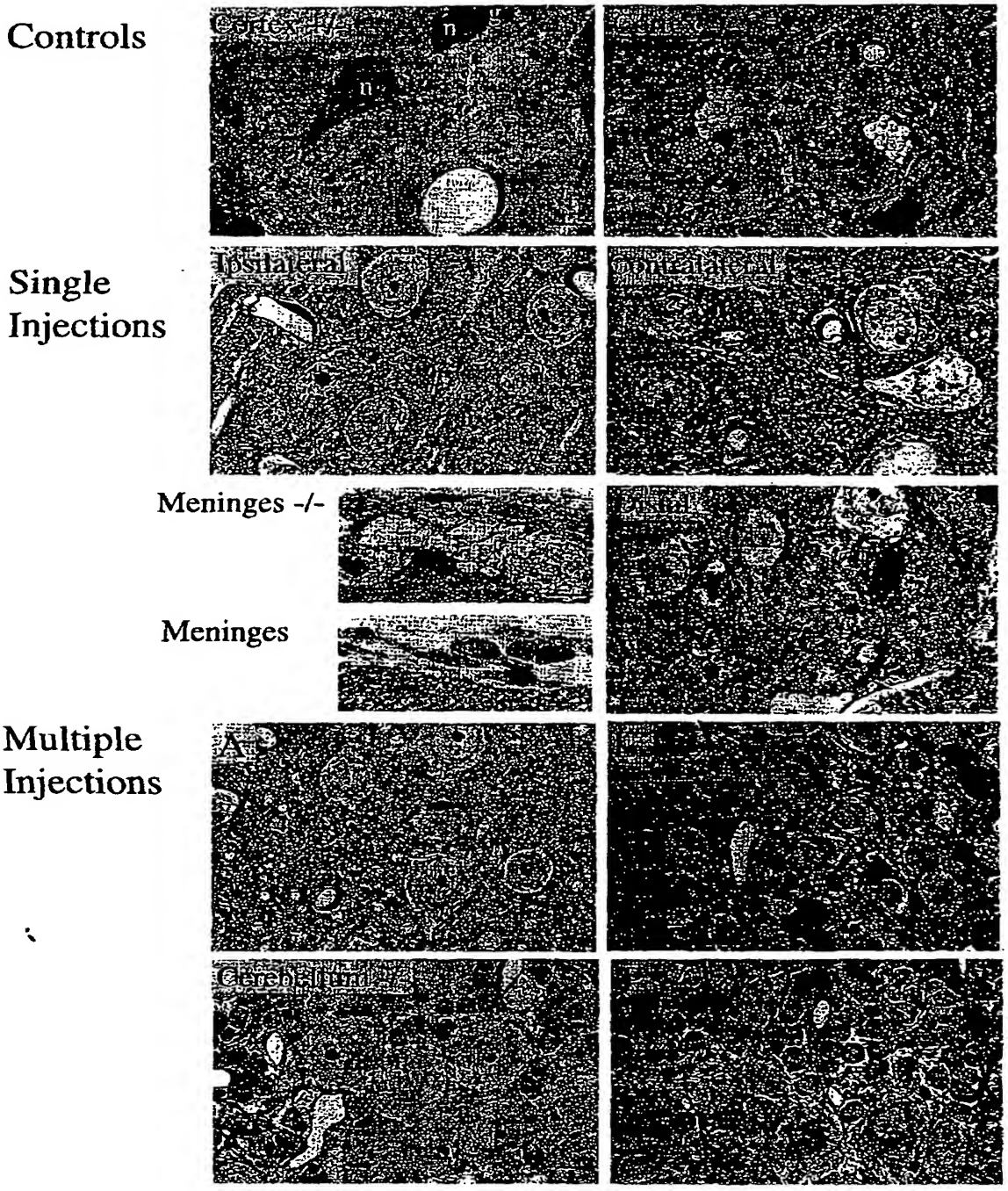

FIG. 6. Correction of lysosomal storage lesions in the brain of treated MPS VII mice. Untreated heterozygote (cortex +/–), untreated MPS VII (cortex –/–, meninges –/–, cerebellum –/–), and MPS VII mice treated with a single intrastriatal or multiple injections of HIVβgluc 16 weeks earlier (all other panels) were killed and used to prepare serial 100-μm sections, as indicated in Figs. 2 and 4. Fragments were randomly picked from sections adjacent to those that had been stained for β-glucuronidase activity, postfixed, and used to prepare 1-μm sections that were stained with toluidine blue. Pictures of sections from an animal treated with a single intrastriatal injection correspond to cortical areas of the injected (ipsilateral, section M in Fig. 2; distal, section D in Fig. 2) and contralateral (contralateral, section M in Fig. 2) hemispheres and to the meninges. Pictures of sections from an animal treated with multiple injections correspond to sections A, L, and P in Fig. 4. n, Neuron; g, glial cells; p, perivascular cell; Pu, Purkinje cell. Lysosomal storage lesions are indicated by arrowheads. Bars = 10 μm.

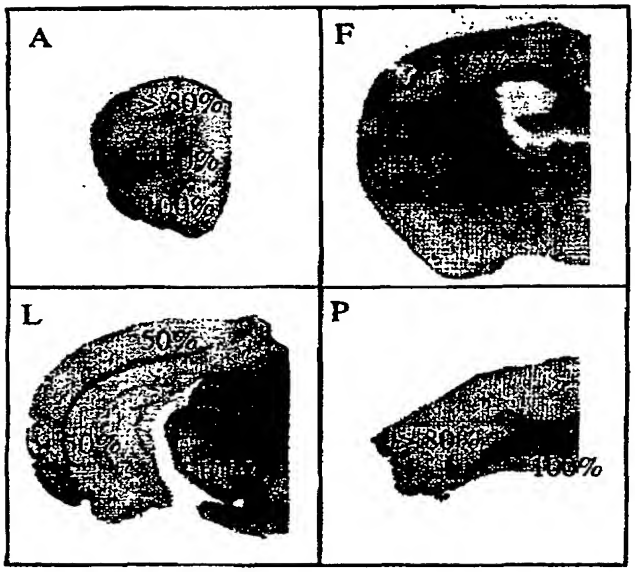

FIG. 7. Estimated level of correction at various locations in the brain of an MPS VII mouse killed 16 weeks after multiple HIVβgluc injections. One-micrometer sections were examined for correction of lysosomal storage lesions, as indicated in Fig. 6. Estimated percentage of correction is indicated at various locations on sections A, F, L, and P (see Fig. 4).

tions. However, more conclusive documentation of enzyme spreading was obtained from histopathology studies than from histochemical staining.

Correction of lysosomal storage lesions

Distension of intracellular vacuoles with glycosaminoglycans is a histopathological hallmark of lysosomal storage diseases. In MPS VII mice, there is a progressive accumulation of lysosomal storage in neurons, glia, and mesenchymal tissues. Lesions are widespread in the brain, including the cortex (Levy *et al.*, 1996). Disappearance of lysosomal storage lesions in response to exogenous enzyme supply has been well documented in peripheral and brain tissues of MPS VII mice (review in Sands *et al.*, 1997b), as well as in the liver of human patients with Gaucher disease (Grabowski *et al.*, 1998). Improvement of histology in brain areas showing β-glucuronidase activity was therefore expected in our experiments. More surprising was the extension of corrected tissues far beyond enzyme-positive areas. Publications by ourselves (Bosch *et al.*, 2000) and by others (Skorupa *et al.*, 1999) report similar findings after AAV-mediated gene transfer. These observations suggest that β-glucuronidase extends further in neural tissue than is detected by histochemistry. The histochemical staining reaction is actually not sensitive, as shown by the almost complete absence of signal in the brain of normal mice. On the other hand, correction of brain damage in areas where little or no enzyme activity can be detected is consistent with observations made in humans with mild forms of MPS I (Scheie's syndrome), which show that enzyme activity representing as little as 0.23% of normal levels is sufficient to prevent the occurrence of neurological symptoms (Ashton *et al.*, 1992).

We carefully assessed the quality and the extent of the correction by picking out samples randomly and regardless of detectable enzyme activity throughout the entire brain of animals treated by multiple injections of HIVβgluc. This study revealed complete or partial disappearance of lesions in every examined sections of the brain at 16 weeks, indicating for the first time that lysosomal storage lesions can be improved in the entire mouse brain. Interestingly, in areas where correction was partial, lesions persisted preferentially in glia and perivascular cells, whereas lysosomal distension had disappeared in most neurons. This strongly suggests that β-glucuronidase can be taken up by neuron prolongations in areas where secretion is intense, then forwarded to remote cell bodies.

Clinical relevance

Clinical benefit could not be assessed in the present experimental design. Indeed, since MPS VII dramatically affects the skeleton and the joints, correction of CNS lesions was unlikely to improve the behavior and life span of MPS VII mice in the absence of a systemic delivery of the enzyme. The death of the animals at the age of 6 months impaired longer observation periods. However, previous studies have shown that enzyme replacement therapy initiated in newborn MPS VII mice and followed by bone marrow transplantation prevents lysosomal storage both in brain and periphery, resulting in a longer life span and better auditory function in treated mice compared with untreated littermates. (Sands *et al.*, 1995; O'Connor *et al.*, 1998). These results demonstrated that enzyme delivery in the whole brain has beneficial clinical consequences in MPS VII mice treated early in life. An important issue with regard to clinical efficacy is to determine to what extent the reversion of pathology, as it could be completely or partially achieved in the entire brain of adult animals, will improve developmental abnormalities. Experiments involving a combination of techniques allowing both systemic and brain delivery of the enzyme are required to investigate this issue.

While this method is potentially therapeutic in human patients, assessment of efficacy in clinical trials in affected children will face various safety concerns. Although accumulation of β-glucuronidase in mouse brain tissues was not associated with any visible toxic effect, a control of gene expression based on the use of an inducible promoter might be required for a regulatable lysosomal enzyme delivery in the brain of human patients. Clinical acceptance of HIV-based vectors is still an issue. The development of vectors based on nonhuman lentiviruses and that of multiply attenuated, self-inactivating vectors will certainly increase acceptability in the future. The need for multiple stereotactic injections in the brain of children may also raise concerns. Increasing gene expression and subsequently the amount of enzyme delivered at each injection site may help to decrease the number of injections to acceptable levels with respect to the expected risks and benefits of gene therapy, the absence of alternative treatment, and the rapidly fatal evolution of most lysosomal storage diseases affecting the CNS.

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